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Award Number: W81XWH-04-1-0491

TITLE: Mechanisms of Chemoresistance in Breast Cancer Cells

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REPORT DATE: May 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-05-2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 May 2005 - 30 Apr 2006	
4. TITLE AND SUBTITLE Mechanisms of Chemoresistance in Breast Cancer Cells				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0491	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Valerie Gouaze, Ph.D.  E-Mail: <a href="mailto:gouazev@jwci.org">gouazev@jwci.org</a>				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  John Wayne Cancer Institute Santa Monica, CA 90049				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT:  The objective of this second year was to determine whether anticancer agents influence the expression of glucosylceramide synthase (GCS). GCS is an enzyme which catalyzes ceramide glycosylation and is associated with chemotherapy resistance in cancer cells. Drugs like doxorubicin, cisplatin, etoposide, and taxol cells had no effect on glucosylceramide (GC) mass production in MCF-7 after short-term exposure (30 min-4 hr). After 24 hr, only taxol induced significant GC production. In short-term experiments using the full-length GCS promoter, C6-ceramide activated GCS after 4 hr treatment, whereas the chemotherapy drugs (epirubicin, idarubicin, and doxorubicin) enhanced GCS promoter activity at 48 hr. When MCF-7 cells were treated with ceramide generating drugs like etoposide, 4-HPR, doxorubicin, or cisplatin for 48 hr, GCS mRNA levels were unchanged compared to untreated cells. Moreover, C6-ceramide did not directly enhance GCS transcription.					
15. SUBJECT TERMS Glucosylceramide synthase, P-glycoprotein, Chemotherapy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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## INTRODUCTION

Poor response to chemotherapy is a major clinical problem, and in most instances drug resistance is underlying cause [1]. This is a most undesirable situation, and patients and oncologists would welcome its possible correction. In breast cancer nearly 50% of patients demonstrate primary and/or secondary resistance to adriamycin (doxorubicin) [2]. Several mechanisms of drug resistance are being examined, and avenues to reverse resistance are being sought. Research strategies in this area have become increasingly aimed at molecular targets such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), topoisomerase, and Bcl-2 protooncogene, to name a few. Overexpression of the membrane efflux transporter, P-gp is one of the most consistent biological alterations in drug resistance [3, 1]. P-gp (170 kDa) is the product of the *MDR1* gene, an energy-dependent pump that reduces the intracellular concentration of specific anticancer drugs, and it has been studied extensively. Our approach to drug resistance is new and involves ceramide metabolism. The area has been reviewed [4, 5]. Several front-line anticancer agents elicit the formation of ceramide, a proapoptotic lipid messenger [6, 7], by activating either the *de novo* or sphingomyelinase pathways of ceramide production [7, 5]. Drugs that induce cellular ceramide generation include anthracyclines (adriamycin), *Vinca* alkaloids, etoposide (VP-16), paclitaxel, and fenretinide (4-HPR). If ceramide formation in response to drug treatment is blocked, then the cytotoxic impact of the drug is largely reduced [8, 9]. This demonstrates ceramide's role in drug responses. Ceramide added directly to cells circumvents the enzyme route of ceramide formation and promotes an apoptotic cascade directly [8, 10]. Our group showed that increased cellular capacity for ceramide glycosylation, catalyzed by glucosylceramide synthase (GCS), is associated with chemotherapy resistance in cancer cells [11-15]. In cultured breast cancer cells, sensitivity to anthracyclines and taxanes can be decreased or increased simply by manipulation of GCS activity [12, 16, 17]. For example, transfection of drug sensitive MCF-7 breast cancer cells with GCS cDNA confers resistance to adriamycin [16], and transfection of multidrug resistance MCF-7-AdrR breast cancer cells with antisense GCS (asGCS) increases cell sensitivity to chemotherapy by a factor of 28-fold for adriamycin, more than 100-fold for vinblastine, and more than 200-fold for paclitaxel [12].

## **BODY**

### -Deviation from the original statement of work

Due to my pregnancy, the experiments using radioactivity to evaluate GC synthesis (in presence or absence of chemotherapeutic drugs) were replaced. Instead, I used GC mass analysis to evaluate GC synthesis. The objective of the second year of the project was not entirely met because of my maternity leave.

## **Key Research Accomplishments**

### **1- Determination of whether GC mass is influenced by adriamycin, etoposide, or taxol treatment.**

MCF-7 cells were treated separately with doxorubicin (2.5  $\mu$ M), cisplatin (CDDP) (10  $\mu$ M), etoposide (0.5  $\mu$ M), and taxol (1.5  $\mu$ M) for 24 hours. Thin-layer chromatographic analysis of total lipid extracts from cells exposed to the chemotherapeutic drugs demonstrated after 30 minutes and 4 hours of treatment that none of the drugs induced an increase in GC mass (Figure 1A). However, after 24 hours, taxol promoted increase of 2-fold in GC mass, compared to MCF-7 cells without taxol (Figure 1B).

### **2- Determination of chemotherapy sensitivity.**

We determined the IC<sub>50</sub> (concentration of drug affecting a 50% kill) of epirubicin and idarubicin in MCF-7 cells. This was essential for subsequent work with the GCS promoter and possible activation by epirubicin and idarubicin.

IC<sub>50</sub> for idarubicin and epirubicin in MCF-7 cells was 0.17 and 0.3  $\mu$ M, respectively (Figure 2).

### **3- Determination of whether anticancer drugs enhance GCS promoter activity.**

MCF-7 cells were transiently transfected with the luciferase-reporter plasmid of the full-length sequence of the human GCS promoter. Cells were then treated with either C6-ceramide (5.0  $\mu$ M), epirubicin (0.5  $\mu$ M), idarubicin (0.3  $\mu$ M), or doxorubicin (0.5  $\mu$ M) for 4 and 48 hr. After 4 hr, luciferase activity in cells treated with C6-ceramide showed a 1.2-fold increase compared to ceramide-naïve cells. Idarubicin, epirubicin, and doxorubicin did not activate the GCS promoter at 4 hr (Figure 3A).

After 48 hours, C6-ceramide, epirubicin, doxorubicin, and idarubicin induced a GCS promoter activation of 4-, 36-, 92.3-, and 316-fold, respectively (Figure 3B).

### **4- Evaluation of GCS mRNA levels after long-term exposure to chemotherapy drugs.**

Initial cell viability experiments were carried out to determine the IC<sub>50</sub> doses for doxorubicin and taxol. Values obtained with doxorubicin and taxol were 0.41 and 0.09  $\mu$ M, respectively (Figure 4), in MCF-7 cells.

MCF-7 cells were treated with ceramide generating drugs such as etoposide, N-(4-hydroxyphenyl) retinamide (4-HPR), doxorubicin, and CDDP. After 48 hr exposure, these drugs failed to induce an increase in expression (Figure 5). This result however does not mean that these drugs do not activate GCS enzymatic activity.

### **5- Determination whether C6-ceramide can act directly to enhance GCS transcription.**

MCF-7 cells were incubated with C6-ceramide (5.0  $\mu$ M) for 48 hr. This short-chain analog of long-chain, natural ceramide is cell permeable and potentiates biological responses seen with ceramide-generating chemotherapeutic agents.

Figure 6 shows that C6-ceramide induced only a 1.12-fold activation of GCS expression. The C6-dihydro-ceramide (C6-di-cer) used as negative control, has no effect on GCS message expression.

#### **Key Research Accomplishments**

- Determined that taxol induced GC production in MCF-7 cells, but not doxorubicin, CDDP, or etoposide.
- Determined IC<sub>50</sub> of epirubicin and idarubicin in MCF-7 cells.
- Determined that C6-ceramide, epirubicin, idarubicin, and doxorubicin activate GCS promoter activity.
- Determined IC<sub>50</sub> of taxol and doxorubicin in MCF-7 cells.
- Determined 4-HPR, etoposide, doxorubicin, and CDDP had no effect on GCS mRNA levels in MCF-7 cells.
- Determined that C6-ceramide and C6-dihydro-ceramide had no effect on GCS mRNA in MCF-7 cells.

## **Reportable Outcome**

### **Poster**

“Relationship between Glucosylceramide synthase and P-glycoprotein in drug resistant human breast cancer cells.” Era of hope 2005, Department of Defense Breast Cancer Research Program. Philadelphia, Pennsylvania, June 8-11, 2005.



## **CONCLUSION**

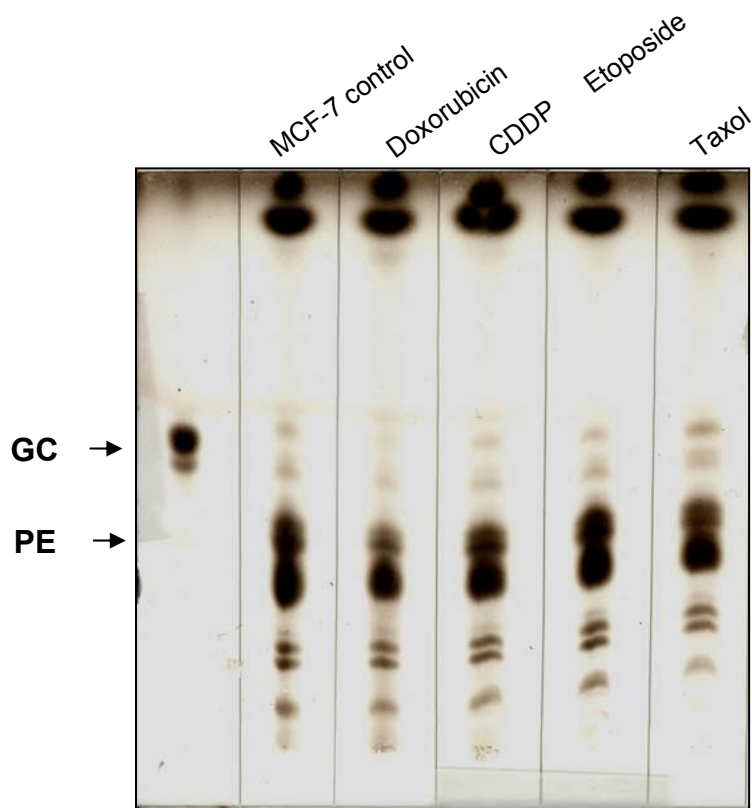
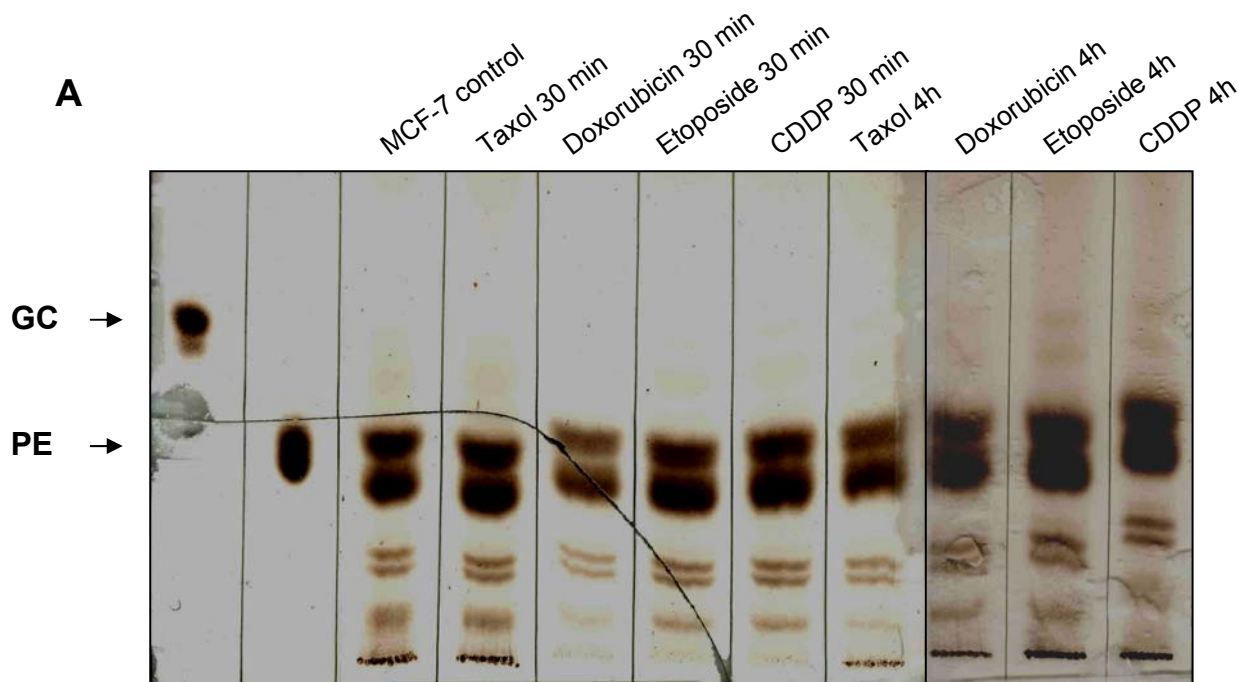
The short-term times experiments showed that chemotherapeutic drugs had no effect on GC production, on GCS promoter activation, or on GCS mRNA levels in MCF-7 cells. The significant GCS promoter activation by chemotherapy at 48 hr suggests that the time frame for modified GCS transcription or GC production by chemotherapy has to be modified and that the influence of chemotherapy drugs on GCS might be a late event.

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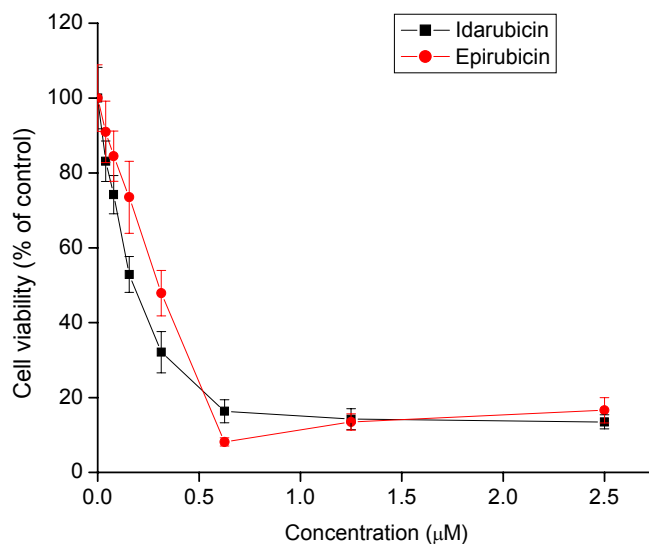
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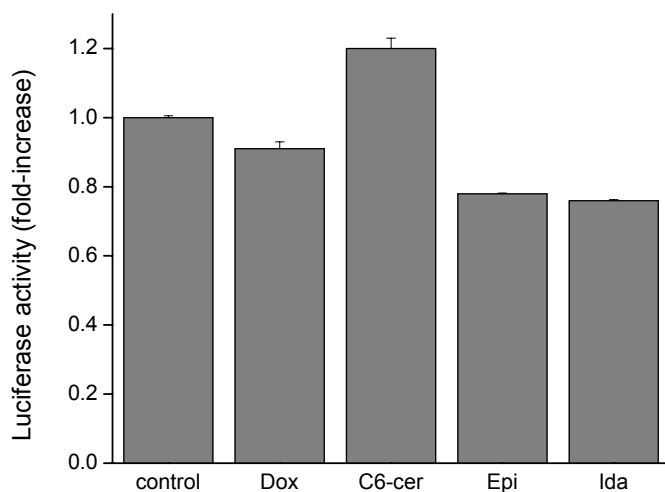
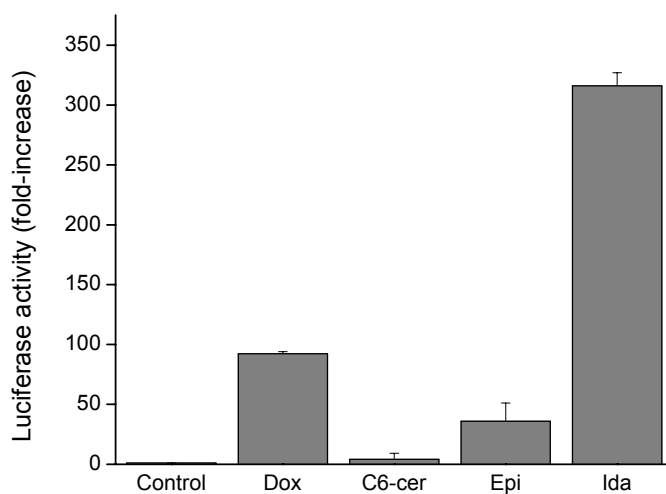
**SUPPORTING DATA**



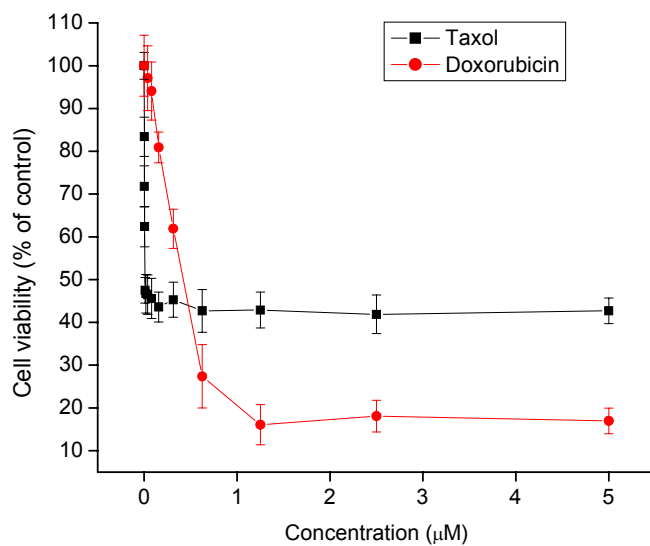
**Figure 1:** Synthesis of GC in MCF-7 cells treated with chemotherapy. **A.** Cells were exposed to doxorubicin (2.5  $\mu$ M), CDDP (10  $\mu$ M), etoposide (0.5  $\mu$ M), Taxol (1.5  $\mu$ M) for 30 min and 4 hr. **B.** Cells were grown in presence of chemotherapy for 24 hr. Total lipids, extracted from washed cells, were analyzed by TLC (297  $\mu$ g lipid/lane for **A**, and 196  $\mu$ g lipid/lane for **B**). The solvent system contained chloroform/methanol/ammonium hydroxide (70:20:4, v/v/v), and visualization was by sulfuric acid char. Commercial standards: GC, glucosylceramide (natural, brain); PE, phosphatidylethanolamine.



**Figure 2:** Cytotoxicity induced by idarubicin, or epirubicin in MCF-7 cells. Cells were exposed to chemotherapeutic drugs at the indicated concentrations for 3 days. Cells viability was determined by MTS assay.

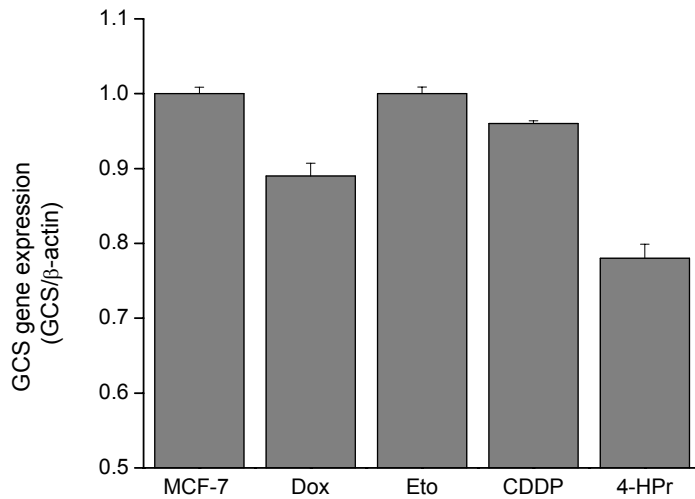
**A****B**

**Figure 3:** GCS promoter activity analysis. After 24 hr transfection with full-length GCS promoter plasmid (pGCS-Luc1, 4  $\mu$ g DNA/well), MCF-7 cells were maintained in 5% FBS RPMI-1640 medium containing doxorubicin (0.5  $\mu$ M), C6-ceramide (5.0  $\mu$ M), epirubicin (0.5  $\mu$ M), or idarubicin (0.3  $\mu$ M) for A, 4hr and B, for 48 hr. Luciferase activity was then determined. The control represents MCF-7 cells transfected with pGCS-Luc1 without treatment.

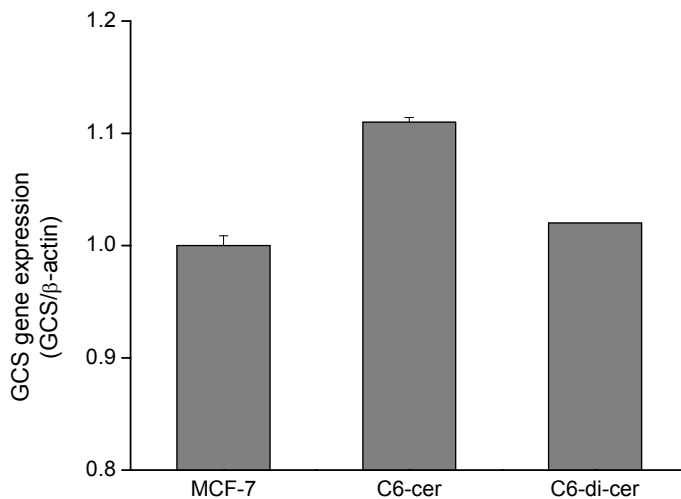


**Figure 4:** Cytotoxicity induced by doxorubicin and taxol in MCF-7 cells. Cells were exposed to chemotherapeutic drugs at the indicated concentrations for 3 days. Cell viability was determined by MTS assay.





**Figure 5:** The influence of anticancer drugs on GCS gene expression. MCF-7 cells were seeded in 6-cm dishes and the following day treated with doxorubicin (2.5  $\mu$ M), etoposide (0.5  $\mu$ M), CDDP (10  $\mu$ M), or-HPR (5.0  $\mu$ M). Control dishes received ethanol (0.1% final concentration). After 48 hr, RNA was extracted and analyzed by real-time RT-PCR.



**Figure 6:** The influence of ceramide on GCS gene expression. MCF-7 cells were treated with C6-ceramide (5.0  $\mu$ M) or C6-dihydroceramide (5.0  $\mu$ M) for 48 hr. RNA was extracted and analyzed by real-time RT-PCR.